STUDIES ON THE HEMORRHAGIC SWEET CLOVER DISEASE

V. IDENTIFICATION AND SYNTHESIS OF THE HEMORRHAGIC AGENT*

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(Received for publication, November 29, 1940)

In this communication it is shown through degradation reactions which lead to the chemical synthesis that the hemorrhagic agent, $C_{19}H_{12}O_6$, m.p. 288–289°, isolated by our colleague, Dr. H. A. Campbell, from spoiled sweet clover hay is 3,3′-methylene-bis(4-hydroxycoumarin), formula (I). Before it was necessary for Dr. Campbell to withdraw from the work due to a change in post, it was concluded that the hemorrhagic agent was not identical with any of the 60 or more naturally occurring coumarins previously reported, and that the literature did not contain an account of a substance melting in the range 280–290° whose chemical properties and analysis could be reconciled with those exhibited by the pure hemorrhagic agent (1, 2).

From a careful study of the chemical and physical properties of the crystalline hemorrhagic agent, it became possible to develop a materially shortened extraction procedure[1] which enabled us to accumulate, by repeated mass isolations in the relatively short

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Cooperative studies with the Division of Forage Crops and Diseases, United States Department of Agriculture, Washington, through Dr. E. A. Hollowell. Personnel and supply assistance since July 1, 1938, in part through the Natural Science Research Project No. 65-1-53-2349 of the Federal Works Progress Administration (Madison).

[1] Inoculation of the atmosphere in the laboratory and the vessels used in the extraction work with the crystals of the hemorrhagic agent probably facilitated the crystallization (3).

period of 4 months, a large stock of the pure substance (1800 mg.). Work on the elucidation of the structure of this unique substance was then undertaken.

The acidic nature of the hemorrhagic agent has been pointed out previously (1, 2). The red color produced with ferric chloride in cyclohexanone, the stability of the dimethyl ether, $C_{19}H_{10}O_4(OCH_3)_2$, toward alkali, and the formation of a diacetate, $C_{19}H_{10}O_6(OCCH_3)_2$, melting point 250–252° with decomposition, suggested that the acidity was due to two enol structures, rather than to free carboxyl groups. The ease with which the compound dissolves in alkali and the nature of the electrometric titration curve (pK 7.2) indicated that the acidity was not due to phenolic hydroxyls or lactone structures. The test for methoxyl and ethoxyl groups was negative. The presence of carbonyl groups was shown by reactions with phenylhydrazine and hydroxylamine.

Insight into the final structure emerged from the degradation experiments that follow. Consideration will be restricted to the crucial reactions having a direct bearing on the structural diagnosis.

Fusion with potassium hydroxide revealed that 14 of the 19 carbon atoms could be recovered as salicylic acid, $C_7H_6O_3$ (III). The yield of salicylic acid proved to be quantitative in view of the final structure deduced for the parent substance (7.3 mg. of the hemorrhagic agent gave 6.1 mg. of salicylic acid). 5 carbon atoms were eliminated by this treatment. Control fusions with 10 mg. quantities of coumarin gave theoretical yields of salicylic acid.

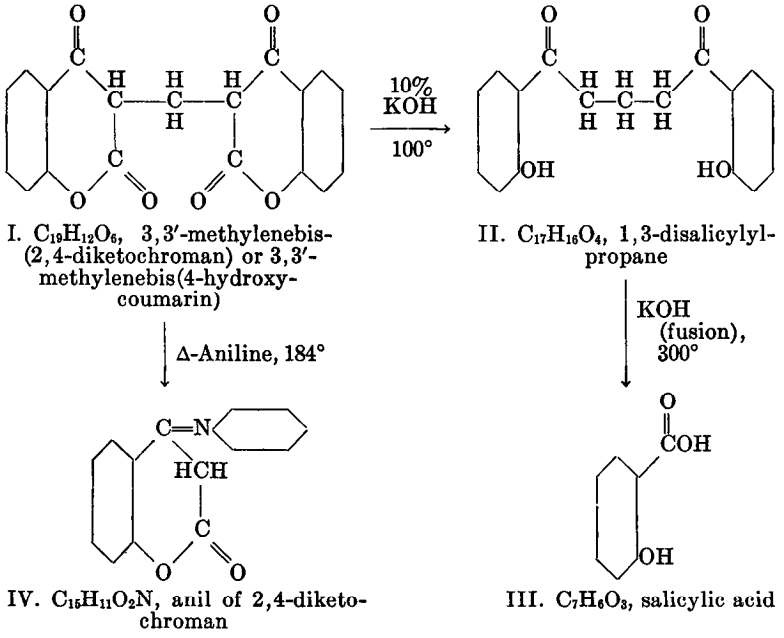
Degradation in 30 per cent alcoholic potassium hydroxide gave some salicylic acid and a new δ-diketone, $C_{17}H_{16}O_4$ (II), m.p. 101–102°, molecular weight 284. The characterization of this δ-diketone as 1,3-disalicylylpropane by two independent synthetic approaches is given in Paper VI of this series (4).

Degradation in 10 per cent sodium hydroxide gave the δ-diketone quantitatively (11.5 mg. of the hemorrhagic agent yielded 9.5 mg. of the δ-diketone). 2 carbon atoms and 2 oxygen atoms were eliminated by this milder alkali treatment.

When viewed collectively, the products isolated from the alkaline degradations indicated that there are present in the hemorrhagic agent, $C_{19}H_{12}O_6$, two benzene nuclei joined through 5 carbon atoms, the carbon bridge of the δ-diketone; that a car-

bonyl oxygen is located on each carbon atom of the bridge adjacent to the benzene rings; and that ortho to the points of attachment is an oxygen atom which is present as a phenol ester. The formation of salicylic acid from the hemorrhagic agent by more drastic alkaline hydrolysis could be rationalized as involving cleavage of the double bonds of the enol form of the intermediate δ-diketone. These products did not establish the position of the 2 carbon and 2 oxygen atoms eliminated in the formation of the δ-diketone.

Degradation of 3,3'-Methylenebis(4-Hydroxycoumarin) under Alkaline Conditions



I. $C_{19}H_{12}O_6$, 3,3'-methylenebis-(2,4-diketochroman) or 3,3'-methylenebis(4-hydroxycoumarin)

II. $C_{17}H_{16}O_4$, 1,3-disalicylylpropane

IV. $C_{16}H_{11}O_2N$, anil of 2,4-diketochroman

III. $C_7H_6O_3$, salicylic acid

However, the ease of formation of the diketone suggests the decarboxylation of a β-keto acid structure. On this basis, together with the absence of phenolic properties in the hemorrhagic agent, the 2 carbon atoms and the 2 oxygen atoms which are eliminated in the formation of the diketone might be assigned to the position β to the carbonyls of the diketone and engaged in lactone formation with the phenolic hydroxyls. This postulation was given support by the following reaction.

Heating the hemorrhagic agent with phenylhydrazine resulted in cleavage to a compound containing 9 carbon atoms ascribable to the parent substance. Analysis of the product, m.p. 189–189.5°, indicated the formula $C_{21}H_{10}O_2N_4$ or $C_9H_4O_2(=N-\overset{H}{N}-C_6H_5)_2$. The recovery of a structural unit containing 9 atoms via degradation with phenylhydrazine suggested that this agent effected a cleavage of the parent substance $C_{19}H_{12}O_6$ into two C_9 units, with a simultaneous loss of 1 carbon atom, and coupling of each of the C_9 units with 2 moles of phenylhydrazine. Precedent for an anomalous reaction of this type under the influence of phenylhydrazine is not common. But this reaction, in conjunction with the results from the alkaline degradations, led us to consider the properties and constitution of the known dicoumarin derivatives, particularly those containing a methylene bridge (5).

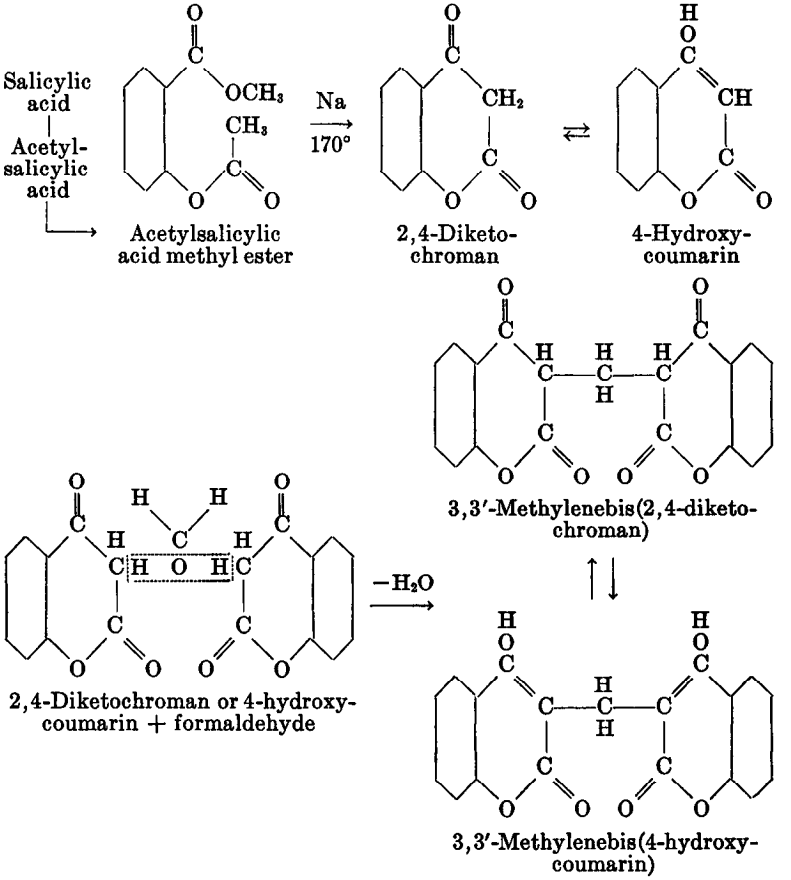
Mature consideration of all the properties exhibited by the hemorrhagic agent and our knowledge of its behavior in the degradation reactions portrayed above placed under strong suspicion the synthetic product, α-methylenebisbenzotetronsäure, $C_{19}H_{12}O_6$, reported by Anschütz in 1903 in conjunction with his classical studies on the benzotetronic acid group (6). The description by Anschütz of this compound was restricted to the melting point, given as 260° (with decomposition), its insolubility in water, solubility in alkali, and recrystallization from benzene. The only reaction mentioned (but without experimental data) was its behavior toward aniline.

When the hemorrhagic agent was heated with aniline, the product formed proved to be the anil of 2,4-diketochroman (4-anilidocoumarin), $C_{15}H_{11}O_2N$, m.p. 262–263° (IV). The melting point of 4-anilidocoumarin reported by Anschütz (6) is 3° below that of our product. 18 of the 19 carbon atoms and all of the oxygen atoms in the hemorrhagic agent could be accounted for by the product recovered from this reaction (13.4 mg. of the hemorrhagic agent gave 18.2 mg. of the anil).

It should be emphasized that the melting point, 288–289°, of the hemorrhagic agent is almost 30° above that reported by Anschütz for the compound listed under the name α-methylenebisbenzotetronsäure. However, all of the degradation reactions of the hemorrhagic agent as well as all of its chemical properties

could be rationalized on the basis of the Anschütz compound. For the physiological activity of the hemorrhagic agent from spoiled sweet clover hay, there existed, however, no parallel (1, 2).

Synthesis of Hemorrhagic Agent from Spoiled Sweet Clover Hay



Accordingly, 4-hydroxycoumarin (benzotetronic acid) prepared from salicylic acid by the method of Pauly (7) was condensed with formaldehyde via the Anschütz procedure (6). No difficulty was experienced in realizing a synthetic product with a melting point in exact agreement with that of the pure naturally occurring hemorrhagic agent, 288–289° All other physical

properties, crystal habit, limited solubility in the common solvents, and the absorption spectrum of the synthetic product were found to be identical with the naturally occurring substance (2). The analytical constants of the dimethyl ether and the diacetate prepared from the synthetic product coincided with those of the corresponding derivatives prepared from the natural material, and the same products (salicylic acid, the δ-diketone, the anil of 2,4-diketochroman) were recovered on degradation.

When the synthetic product was fed to our standardized assay rabbits at the levels previously reported for the natural substance, a comparable reduction in prothrombin level (or activity) was observed (1, 2, 8). Continued feeding of the synthetic product to rabbits, rats, guinea pigs, and dogs produced the fatal hemorrhages characteristic of the sweet clover disease (9, 10).

Since the origin of this exceptional substance in the spoiled sweet clover hays is the coumarin molecule, we prefer to have it designated as a derivative of coumarin, and accordingly suggest the use of the name 3,3′-methylenebis(4-hydroxycoumarin). It is apparent that tautomeric modification of 3,3′-methylenebis(4-hydroxycoumarin) into 3,3′-methylenebis(2,4-diketochroman) is possible. To this reversible transformation is ascribed, *inter alia*, the acidic properties of the substance, its behavior toward carbonyl reagents, and its degradation by alkali. Thus salt formation, methylation, and acetylation of the enol form of the substance would be expected. The cleavage caused by the organic bases phenylhydrazine and aniline may be considered a reverse aldol condensation. The mild alkali treatments effect opening of the lactone rings to produce a β-keto acid, which would decarboxylate readily to the δ-diketone. Stronger alkali effects cleavage of the double bond of the enol form of the diketone to produce salicylic acid (4). Accordingly in the formulations indicating the synthesis of 4-hydroxycoumarin and 3,3′-methylenebis(4-hydroxycoumarin) the keto structures are also indicated.

EXPERIMENTAL

Mass Isolation of Hemorrhagic Agent (*1*, *2*)—3 kilos of the spoiled hay were extracted with 30 liters of water at pH 3, steeped in 0.1 N sodium hydroxide, acidified to pH 3, filtered, and the residue extracted with two 20 liter portions of ethyl alcohol. The

alcoholic extract from 9 kilos of hay was concentrated at 25°, and the residue dissolved in 0.5 per cent sodium hydroxide and then acidified to pH 3. The precipitate was collected, suspended in 1 liter of methanol, and 2 liters of ethyl ether added. After filtration the methyl alcohol was removed by shaking with 6 liters of 2 per cent hydrochloric acid. The green ether solution was shaken with 36 per cent hydrochloric acid until the acid layer was just slightly colored. During this treatment additional ether was added to maintain the ether volume above 2 liters. The ether was concentrated to 500 ml. at atmospheric pressure, then to a thin syrup at reduced pressure. On examination in polarized light under high magnification, this syrup showed the presence of microscopic crystals of the hemorrhagic agent embedded in a matrix of amorphous material. Solubility tests indicated that the inert materials could be removed selectively.

The syrup was suspended by shaking with 200 ml. of methanol and centrifuged. The methanol layer was decanted and the solids resuspended in 200 ml. of methanol by shaking. This operation was repeated until the methanol washings were only slightly colored.

The remaining solids were transferred to a 50 ml. centrifuge tube and washed with a 10 per cent solution of Skellysolve A in methanol. This operation was repeated several times with methanol containing increasing concentrations of Skellysolve A. The progress of the washing was followed by observing the appearance of the crystals under polarized light. Recrystallization from benzene or cyclohexanone yielded macroscopic crystals of the crude product. After the final recrystallization from cyclohexanone the melting point of 288–289° was obtained. Thirty successive extractions gave 1800 mg. of the pure hemorrhagic agent, the over-all recovery (based on the prothrombin assays) being between 66 and 73 per cent of the total quantity present.

Preparation of Diacetate, $C_{19}H_{10}O_6(OCCH_3)_2$—The hemorrhagic agent (28.3 mg.) was added to 1 ml. of a mixture of equal parts of pyridine and acetic anhydride at 28°. After 30 minutes the crude acetate was filtered off, pressed dry, and recrystallized from benzene. The acetate melts at 250–252° (decomposes). Yield 34.7 mg. or 98 per cent of theory.

Analysis of Diacetate—The acetyl groups could not be estimated

with the usual acid saponification procedures. But after saponification in alcoholic potassium hydroxide and acidification with concentrated sulfuric acid, successful estimations were realized in the Perkins apparatus.

$C_{19}H_{10}O_6(OCCH_3)_2$

Calculated.	Acetyl 20.48,	C 65.71,	H 3.81
Found.	" 20.4, 20.0,	" 65.80, 65.72,	" 3.89, 3.81

Alkaline Degradation of Hemorrhagic Agent—In each of the three degradation conditions given below the reaction mixture was worked up by the usual procedures for neutral, acidic, and phenolic substances. The over-all procedure will be apparent from the fusion with solid potassium hydroxide (*a*).

(*a*) *Degradation with Solid Potassium Hydroxide*—The hemorrhagic agent (7.3 mg.) was heated with 0.5 gm. of potassium hydroxide in a nickel crucible at 300° until the starting product dissolved with the evolution of gas. The melt was then held at approximately 300° for 10 minutes. The fused mass was cooled and taken up in 5 ml. of water.

The basic solution was first extracted with 5 ml. portions of ethyl ether to remove the neutral products. These were nil. The ether-extracted aqueous solution was then made acid with concentrated hydrochloric acid and again extracted with ethyl ether. The ether solution was extracted with 10 per cent sodium bicarbonate (5 ml.). The bicarbonate solution was acidified and extracted with ethyl ether. The resulting ether extract was washed with water, and then concentrated to permit crystallization of the acidic fragments. Only salicylic acid, $C_7H_6O_3$, was recovered, which was characterized by its melting point, 158–159°, and methyl ester. Yield 6.1 mg. or 98 per cent of theory. Under the same conditions control experiments with 10 mg. of coumarin gave salicylic acid quantitatively.

The ether solution remaining after the bicarbonate extraction was washed with water and concentrated to dryness. The quantity of phenolic material was negligible.

(*b*) *Degradation with 30 Per Cent Alcoholic Potassium Hydroxide*—The hemorrhagic agent (6.2 mg.) was refluxed in 5 ml. of 30 per cent potassium hydroxide in 90 per cent methyl alcohol for 24 hours. The reaction mixture was worked up as given in

section (*a*). The neutral fraction was nil. The phenolic fraction yielded 4.0 mg. of a δ-diketone with a melting point of 98–100°, which rose to 101–102° after recrystallization from 95 per cent ethanol. Since this product appears again in degradation (*c*), further comment on it will be deferred.

The acidic fraction yielded 2.5 mg. of salicylic acid (m.p. 157–158°).

(*c*) *Degradation with Aqueous Sodium Hydroxide*—The hemorrhagic agent (11.5 mg.) was refluxed in 10 per cent aqueous sodium hydroxide for 40 hours. The neutral and acidic fractions were nil. The phenolic fraction gave 9.5 mg. of the δ-diketone, 1,3-disalicylylpropane, $C_{17}H_{16}O_4$, m.p. 101–102°. Yield over 95 per cent of theory.

Properties of δ-Diketone (*4*)—It is optically inactive, the ferric chloride test is positive in ethanol, and the Folin-Denis test is also positive. The ketone couples with diazotized *p*-nitraniline (red precipitate).

Analysis of δ-Diketone—

$C_{17}H_{16}O_4$.	Calculated.	C 71.83,	H 5.63,	mol. wt. 284
	Found.	" 71.75, 71.70,	" 5.53, 5.60,	" " 280
		(micro-Rast in camphor)		(average)

The δ-diketone forms a diether, $C_{17}H_{14}O_2(OCH_3)_2$, m.p. 86–88° On fusion with potassium hydroxide the diketone yields salicylic acid quantitatively. It therefore represents an intermediate degradation product between the hemorrhagic agent and salicylic acid. Details of the characterization of the δ-diketone as 1,3-disalicylylpropane through synthesis by two independent methods are given in Paper VI of this series (4).

Degradation of Hemorrhagic Agent by Heating in Aniline—The hemorrhagic agent (13.4 mg.) was heated with redistilled aniline (0.2 ml.) at 180° for 30 minutes. The reaction mixture was poured into dilute hydrochloric acid to dissolve the excess aniline. The crude anil (18.2 mg.) was filtered and washed first with dilute acid and then with water. After one recrystallization from 20 ml. of 95 per cent ethanol the melting point rose to 262–263°, which is 3° above the melting point reported by Anschütz (6) for the anil of 2,4-diketochroman (4-anilidocoumarin). Yield approximately 95 per cent of theory. A control synthesis of the anil

from 4-hydroxycoumarin was made on a macro scale. The products have identical properties and composition.

Analysis of Anil—

$C_{15}H_{11}O_2N$.	Calculated.	N 5.83,	C 75.95,	H 4.64
	Found.	" 5.78, 5.90,	" 76.00, 76.03,	" 4.70, 4.73

Degradation of Hemorrhagic Agent by Heating with Phenylhydrazine—The hemorrhagic agent (13.0 mg.) dissolved in 0.5 ml. of phenylhydrazine was heated at 135° for 30 minutes. The reaction mixture was poured into 10 per cent hydrochloric acid, whereupon a red gummy oil separated, which was washed by decantation with the acid. The reaction product was dissolved in ethanol and recrystallized (two times). Yield 13.9 mg., m.p. 189–189.5°.

The same product was also made in a control synthesis from 4-hydroxycoumarin on a macro scale. Anschütz (6) reported that three different products are realized when 4-hydroxycoumarin is heated with phenylhydrazine. He listed one with a melting point of 186° containing 11.05 per cent nitrogen (one phenylhydrazine residue). It appears from the analyses reported below that the product realized by us contains two phenylhydrazine residues, which might occupy positions 3 and 4 in the coumarin ring.

Analysis of Phenylhydrazine Derivative—$C_{21}H_{16}O_2N_4$

Calculated.	N 15.75,	C 70.75,	H 4.49,	mol. wt.	356
Found.	" 15.74, 15.68,	" 70.60, 70.70,	" 4.54, 4.59,	" "	364

(micro-Rast in camphor)

Synthesis of 4-Hydroxycoumarin (Benzotetronic Acid) (7)—Acetyl methylsalicylate (100 gm.) was heated to 165° on an oil bath in an open beaker. Metallic sodium (12 gm.) was introduced into the melt (with stirring) over a period of 1 hour. The temperature was maintained between 165–175° by cooling. As the sodium compound separated, the melt thickened and finally solidified. The mass was then ground, the unused sodium decomposed with methanol, and the product taken up in 500 ml. of water. After filtration the 4-hydroxycoumarin was precipitated by acidifying with hydrochloric acid. The product was washed with dilute acid, and recrystallized from hot water. Yield 12 gm. (14 per cent of theory), m.p. 204–206°. Repeated recrystallization raised the melting point to 210°.

Synthesis of 3,3'-Methylenebis(4-Hydroxycoumarin) (6)—5.0

gm. of 4-hydroxycoumarin were dissolved in 1.5 liters of hot water (99°) to which an excess of 40 per cent formalin was added. The crude product was collected and washed copiously with water. It was dried and recrystallized from cyclohexanone, m.p. 288–289°. Yield 4.7 gm. (91 per cent of theory). The melting point of a

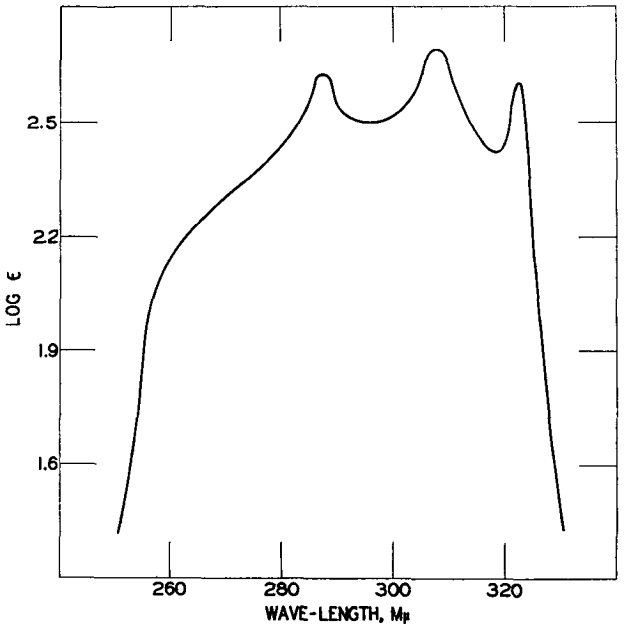


FIG. 1. Absorption spectrum of 3,3′-methylenebis(4-hydroxycoumarin) in cyclohexane; absorption maxima in mμ (log extinction values in parentheses), 288.0 (2.628), 308.5 (2.689), 323.5 (2.608).

mixture of the synthetic product and that isolated from hemorrhagic hay was 288–289°.

Analysis of Synthetic Product—$C_{19}H_{12}O_6$

Calculated.	C 67.80,	H 3.60,	O 28.60
Found.	" 67.84, 67.80,	" 3.49, 3.53,	" 28.67 (by difference)

Electrometric Titration—The curve obtained with the synthetic product was so close to that of the natural product that the details need not be given again (2).

Absorption Spectra—The absorption curves obtained with the natural and the synthetic product were identical (see Fig. 1).

Physiological Activity (*2, 8*)—1.5 mg. doses of 3,3′-methylenebis(4-hydroxycoumarin) fed to standardized susceptible rabbits (with relative clotting index values[2] of about 0.30 at the time of standardization) gave an index of about 0.50 after 24 hours and a relative clotting index of less than 0.10 after 40 hours, representative of an increase in prothrombin time from 25 to 28 seconds to 85 to 90 seconds. Continued feeding of the synthetic product to rabbits, rats, guinea pigs, and dogs first produced a prolonged prothrombin and whole blood clotting time followed by the hemorrhagic condition characteristic of the sweet clover disease, which eventually became fatal (9, 10). The details on the physiological activity of the substance at different dosage levels with various animals will be considered in a separate communication.

DISCUSSION

Relation of This Study to Development of Low Coumarin Strains of Sweet Clover—In previous publications from this station (11–14) it was pointed out that both of the undesirable aspects of sweet clover as a cultivated crop,[3] its unpalatability (bitterness) and the tendency of the hays to become hemorrhagic when improperly cured, appeared to have a common basis in the coumarin molecule. The elucidation of the structure of the hemorrhagic agent as 3,3′-methylenebis(4-hydroxycoumarin) substantiates this relationship, and suggests that the biological synthesis during spoilage involves an oxidation of the coumarin to 4-hydroxycoumarin followed by coupling with formaldehyde.

On this basis, the amount of coumarin in the sweet clover which was converted to the hemorrhagic agent in the spoiled hays employed in this study (1, 2) would be 0.0026 per cent (dry substance basis). The final coumarin content of these spoiled hays ranged from 0.75 to 1.58 per cent, which indicates that only a very small amount of the total coumarin was involved.

Reduction of the coumarin content by breeding or selection

[2] The relative clotting index is the ratio of the concentration of the normal plasma in the concentration range of 12.5 to 8.34 per cent to the concentration of the pathic plasma which gives the same clotting time (8).

[3] During the last three decades sweet clover in North America has risen from the status of a roadside weed to a place of importance as a forage crop (15).

(11, 12, 16) offers the possibility of improving palatability and reducing the economic hazard associated with the feeding of spoiled sweet clover hays or silage. But in view of the small amount of coumarin actually involved in the formation of hemorrhagic hays, and the limitations of the methods available at present for the estimation of coumarin (17), any strains of sweet clover selected by the plant breeder on the basis of the coumarin content as potentially desirable should be checked through actual spoilage tests, in which the prothrombin assay with standardized susceptible rabbits is used as the control determination (8).

3,3′-Methylenebis(4-Hydroxycoumarin) and Blood Coagulation Problem—Roderick pointed out that the hemorrhagic sweet clover disease is in a sense without parallel in animal pathology or human medicine (9, 10). Recently Quick (18) has affirmed this view in a résumé on the classification of hemorrhagic diseases due to defects in the coagulation mechanism of the blood and suggested that similar toxic agents may perhaps be encountered clinically which may attack the prothrombin of the blood.[4]

We should like to call attention to certain observations made with 3,3′-methylenebis(4-hydroxycoumarin) which might interest those dealing with the general problem of blood coagulation. The syndrome produced by feeding the substance to rabbits apparently does not result in permanent injury. Some of our assay rabbits have had the prothrombin level (or activity) reduced to 10 per cent, or below, of the normal over 100 times (10 day rest period between assays) without developing either indications of permanent injury, the acquisition of immunity, or increased susceptibility to the hemorrhagic agent (8). The administration of massive single doses (1.0 gm. to a 2.5 kilo rabbit or 5.0 gm. to an 8 kilo dog) effected a reduction in the prothrombin level (or activity) without producing gross signs of permanent injury. It appears that continued feeding of the substance is necessary for the production of hemorrhages.

In view of the prothrombin-reducing or inactivating properties of this dicoumarin, and the spread between the detectable and lethal dose, together with the relative ease with which it may be synthesized and administered, it would appear that its anticoagu-

[4] See also the recent comprehensive review on plasma prothrombin and vitamin K by Brinkhous (19).

lant properties merit consideration from the physiologist and hematologist (19, 20).

SUMMARY

1. Proof is presented through degradation reactions and by synthesis, that the hemorrhagic agent, $C_{19}H_{12}O_6$, m.p. 288–289°, present in improperly cured hay made from the common sweet clovers, *Melilotus alba* and *Melilotus officinalis*, is the dicoumarin, 3,3′-methylenebis(4-hydroxycoumarin).

2. The chemical and physical properties of the naturally occurring and the synthetic products have been shown to be identical.

3. The synthetic product has been shown to parallel the naturally occurring product in its capacity to reduce the prothrombin level (or activity) of standardized susceptible rabbits.

4. The hemorrhagic condition characteristic of the sweet clover disease has been produced in various species of experimental animals by continued feeding of the synthetic 3,3′-methylenebis(4-hydroxycoumarin).

5. The bearing of this study on the objectives of the plant geneticist to breed a low coumarin line of sweet clover and the possible value of the hemorrhagic agent to the physiologist and hematologist are discussed.

We are indebted to our colleagues, Ralph S. Overman for the prothrombin assays, William Sullivan for the acetyl determinations, and Dr. R. J. Dimler for the micro-Dumas nitrogen determinations and for assistance in the preparation of the manuscript.

BIBLIOGRAPHY

1. Campbell, H. A., Roberts, W. L., Smith, W. K., and Link, K. P., *J. Biol. Chem.*, **136**, 47 (1940).
2. Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, **138**, 21 (1941).
3. Tutton, A. E., The natural history of crystals, London, 42 (1924).
4. Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, **138**, 529 (1941).
5. Simonis, H., Die Cumarine, Stuttgart (1916).
6. Anschütz, R., *Ber. chem. Ges.*, **36**, 465 (1903); *Ann. Chem.*, **367**, 169 (1909).
7. Pauly, H., and Lockemann, K., *Ber. chem. Ges.*, **48**, 28 (1915).
8. Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, **138**, 1 (1941).
9. Roderick, L. M., *J. Am. Vet. Med. Assn.*, **74**, 314 (1929); *Am. J. Physiol.*, **96**, 413 (1931).

10. Roderick, L. M., and Schalk, A. F., *North Dakota Agric. Exp. Stat., Bull. 250* (1931).
11. Brink, R. A., *Science*, **79**, 301 (1934).
12. Brink, R. A., and Roberts, W. L., *Science*, **86**, 41 (1937).
13. Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, **119**, 269 (1937); *Ind. and Eng. Chem., Anal. Ed.*, **9**, 438 (1937).
14. Smith, W. K., and Brink, R. A., *J. Agric. Research*, **57**, 145 (1938).
15. *U. S. Dept. Agric., Bur. Plant Ind., Bull. 1653* (1939).
16. Stevenson, T. M., and White, W. J., *Scient. Agric.*, **21**, 18 (1940).
17. White, W. J., and Horner, W. W., *Scient. Agric.*, **21**, 29 (1940).
18. Quick, A. J., *Am. J. Med. Sc.*, **199**, 118 (1940).
19. Brinkhous, K. M., *Medicine*, **19**, 329 (1940).
20. Eagle, H., A symposium on blood and the blood-forming organs, Madison, 242–264 (1940).